In vivo Imaging of Cerebral ‘Peripheral Benzodiazepine Binding Sites’ in Patients with Hepatic Encephalopathy

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Abstract
Background and Aim: One proposed mechanism whereby hepatic encephalopathy (HE) leads to a loss of brain function is the dysregulated synthesis of neurosteroids. The mitochondrial synthesis of neurosteroids is regulated by ‘peripheral benzodiazepine binding sites’ (PBBS). Expressed in the brain by activated glial cells, PBBS can be measured in vivo by the specific ligand $^{[11}C](R)$-PK11195 and positron emission tomography (PET). Recently, it has been suggested that PBBS-expressing glial cells may play a role in the general inflammatory responses seen in HE. We, therefore, measured PBBS in vivo in the brains of patients with minimal HE using $^{[11}C](R)$-PK11195 PET.

Methods: Five patients with minimal HE and biopsy-proven cirrhosis of differing aetiology were assessed with a neuropsychometric battery. The regional expression of PBBS in the brain was detected by $^{[11}C](R)$-PK11195 PET.

Results: All patients showed brain regions with increased $^{[11}C](R)$-PK11195 binding. Significant increases in glial $^{[11}C](R)$-PK11195 binding were found bilaterally in the pallidum, the right putamen and right dorsolateral prefrontal region. The patient with the most severe cognitive impairment had the highest increases in regional $^{[11}C](R)$-PK11195 binding.

Conclusion: HE is associated with increased cerebral binding of $^{[11}C](R)$-PK11195 in vivo, reflecting an increased expression of PBBS by glial cells. This supports earlier experimental evidence in rodent models of liver failure, suggesting that an altered glial cell state, as evidenced by the increase in cerebral PBBS, might be causally related to impaired brain functioning in HE.
Introduction

Chronic hepatic encephalopathy (HE) is a characteristically reversible neuropsychiatric disorder without overt structural brain damage, which occurs on the background of chronic liver disease. Minimal HE is characterised by a subtle impairment of cognitive function, leading to deficits in attention and impaired reaction times in driving or operating machinery, whereas confusion, stupor and coma are the manifestations of overt disease.[1]

One change observed consistently in the brain of mice with induced hyperammonemia[2] or in post-mortem brain tissues of patients with portal-systemic encephalopathy [3] is a significant increase in the expression of “peripheral benzodiazepine binding sites” (PBBS). Originally discovered as additional binding sites of certain benzodiazepines, such as diazepam, PBBS is a heteromeric complex that is largely, though not exclusively, localised in the outer mitochondrial membrane.[4] PBBS was, however, found to be structurally and functionally unrelated to the central benzodiazepine receptor, which is associated with gamma-aminobutyric acid (GABA)-regulated channels. Subsequently, the isoquinoline PK11195, a specific high-affinity ligand, has been used to pharmacologically characterise PBBS.[5][6] Therefore, we will refer to PBBS either as ‘PK11195 binding (sites)’ or ‘(R)-PK11195 binding’, where the data are obtained using the R-enantiomer, for which a slightly higher affinity for the PBBS has been reported.[7]

Unlike in peripheral organs and cell types, normal brain parenchyma has only minimal binding of PK11195. However, as soon as brain disease gives rise to active tissue pathology, a de novo expression of PK11195 binding sites is observed. At least three cellular sources have been discussed: (a) astrocytes.[3][8] (b) invading blood-borne cells of mononuclear-macrophage lineage, if the blood-brain barrier is disrupted [9] or c) activated microglia, the intrinsic population of the normally dormant brain macrophages, in those conditions where the blood-brain barrier has remained intact.[10][11][12] A role of PBBS in the pathogenesis of HE has been hypothesised, based on their regulatory effect on mitochondrial cholesterol transport and thus the altered synthesis of neurosteroids, which may be responsible for neuronal inhibition observed in HE.[6][13]

Additionally, recent evidence points to systemic inflammatory immune responses in minimal HE as important mediators or at least enhancers of ammonia toxicity.[14][15] The fact that glial cells, notably microglia, the dominant immune effector cell of the brain, can rapidly express PBBS de novo in response to even the most subtle pathological stimuli is thus of added significance.[10][11][12][15] The exclusively non-neuronal PBBS directly regulate many immune functions, such as release of cytokines and reactive oxygen intermediates and are strongly regulated even in pathological conditions that are not characterised by overt tissue damage or inflammatory signs, such as recruitment of blood-borne immune cells.[11] There is, therefore, the intriguing possibility, that the PBBS-expressing glial cells are the local participants in the general inflammatory responses seen in HE. Labelled with carbon-11, (R)-PK11195 is a ligand for the measurement of PK11195 binding for PBBS in vivo by positron emission tomography (PET). To address the fundamental question of whether there is a change in the PBBS expression in the brains of patients with minimal HE, we report the measurement of cerebral [11C](R)-PK11195 binding in 5 patients with HE and biopsy-proven cirrhosis of differing etiology. We relate the regional pattern of increased [11C](R)-PK11195 binding to the underlying severity of liver disease and psychometric test analysis.
Subjects and Methods

Subjects

Five patients with HE with biopsy-proven cirrhosis (mean $\pm SD$ age 60.4 $\pm 15.4$ years) were recruited for the study. The patient cohort comprised two patients with alcohol-related cirrhosis, one with autoimmune chronic active hepatitis/cirrhosis, one with post-viral cirrhosis secondary to hepatitis C virus (HCV) infection and one with a dual HCV and alcohol-related cirrhosis aetiology. The patient with autoimmune chronic active hepatitis/cirrhosis (Table 1, patient 1) had a surgically-placed portocaval shunt of more than 20 years duration and a history of well documented minimal HE. This patient was taking maintenance oral prednisolone at 4-5mg daily with intermittent courses of azathioprine over this 20 yr period. All patients were clinically stable at the time of the study and had been abstinent from alcohol for a minimum of 6 months prior to the PET examination. None was receiving psychoactive medication and none was thiamine deficient. Patients with a prior history of alcohol excess had started thiamine supplementation at the time of first diagnosis.

Blood was drawn for standard biochemical and haematological parameters of liver function and for serum electrolytes. A Pugh's score and Child's grade (as modified by Pugh)[16] reflecting the severity of hepatic dysfunction were calculated for each subject (Table 1). Functionally, three were Child's grade A, one Child’s grade B and one was classified as having Child's grade C disease (Table 1).
### Table 1 Summary of the clinical characteristics for each patient with hepatic encephalopathy (HE).

#### Clinical data for patients studied

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age</th>
<th>Gender</th>
<th>Etiology</th>
<th>Disease duration [years]</th>
<th>CHILD</th>
<th>Bilirubin [µmol/L]</th>
<th>Albumin [g/L]</th>
<th>NH₃ [µmol/L]</th>
<th>Varices</th>
<th>Surgical shunt [years]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>57</td>
<td>F</td>
<td>autoimmune</td>
<td>22</td>
<td>B</td>
<td>72</td>
<td>34</td>
<td>91</td>
<td>-</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>F</td>
<td>alcohol</td>
<td>4</td>
<td>A</td>
<td>8</td>
<td>41</td>
<td>286</td>
<td>-</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>78</td>
<td>F</td>
<td>hepatitis C</td>
<td>14</td>
<td>A</td>
<td>22</td>
<td>37</td>
<td>75</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>M</td>
<td>hepatitis C / alcohol</td>
<td>3</td>
<td>A</td>
<td>16</td>
<td>44</td>
<td>181</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>M</td>
<td>alcohol</td>
<td>10</td>
<td>C</td>
<td>31</td>
<td>24</td>
<td>57</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N=10</td>
<td>62.7</td>
<td>6F / 4M</td>
<td></td>
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</table>

- a) surgical portocaval anastomosis
- b) TIPSS shunt
A full neurological, psychometric and electrophysiological assessment was performed on each patient. The mental state was assessed using West Haven criteria.[17] Psychometric performance was assessed under standardised conditions, using a battery of four tests comprising Number Connection Tests (NCT) A and B[18], the Digit Symbol subtest of the Wechsler Adult Intelligence Scale (WAIS)[19] and the Digit Copying subscore of the Kendrick battery.[20] Results are shown in Table 2.

Table 2
The results of four psychometric tests are reported for individual patients. The normal range derives from a data-set obtained from a cohort of healthy age-matched controls.

<table>
<thead>
<tr>
<th>Patients</th>
<th>NCT A (sec)</th>
<th>NCT B (sec)</th>
<th>Digit Symbol</th>
<th>Digit Copying</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>100</td>
<td>76</td>
<td>128</td>
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<tr>
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<td>50</td>
<td>154</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>not completed</td>
<td>45</td>
<td>118</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>90</td>
<td>67</td>
<td>143</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>not completed</td>
<td>19</td>
<td>80</td>
</tr>
</tbody>
</table>

Normal range 15-37 sec 31-81 sec 55-90 124-208

Electroencephalograms (EEG) were performed using conventionally placed electrodes and the mean cycle frequency was obtained. Capillary ammonia concentrations from a finger prick were measured from the pulp of the index finger, using a Blood Ammonia Checker II (Kyoto Daichichi Kagaku Co, Ltd, Kyoto, Japan) All patients underwent baseline T1-weighted volumetric magnetic resonance imaging (MRI) on the same day of the PET study with $[^{11}\text{C}]$(R)-PK11195 to rule out systemic significant structural brain pathology. Additionally, patients were characterised within one week of the PET study by in vivo cerebral $^1\text{H}$ MRS and $^{31}\text{P}$ MRS. The methods of data acquisition and analysis have previously been reported.[21][22][23] Exclusion criteria for PET study were the presence of focal brain lesions or severe atrophy detected by MRI, the presence of a past history of neurological or psychiatric disorders, episodes of acute HE or gastrointestinal bleeding in the previous three months.

The cohort of control subjects enrolled in the PET study consisted of 10 age-matched healthy volunteers (mean [±SD] age 62.7 [±12.7] years), the age range was from 41 to 80 years, comparable to the mean and age range of the patients studied. Also, except for the thalamus, there is no region in the brain in which a significant age-related increase of baseline binding of $[^{11}\text{C}]$(R)-PK11195 has been observed.[24] Each subject underwent an extensive clinical, neurological and psychometric investigation and a T1-weighted volumetric MRI scan the same day of the PET study with $[^{11}\text{C}]$(R)-PK11195 to rule out systemic and neurological diseases.
The study conformed to the guidelines set out in the Declaration of Helsinki of 1975 and prior ethical approval was obtained from the Ethics Committee of the Imperial College School of Medicine, London. All subjects provided written informed consent.

**PET study**

The PET study was performed on a CTI/Siemens ECAT 953B PET scanner operated in 3D acquisition mode. $[^{11}\text{C}]$(R)-PK11195 was injected as a bolus 30s after the acquisition scan started. The mean tracer dose was 360±30 MBq with a specific activity of 37±1 GBq/mmol. Dynamic data were collected over 60 min as 18 temporal frames. Attenuation correction factors were determined using a 15 min transmission scan acquired before the dynamic scan. Scatter correction was achieved using a dual-energy window method.[25] Data were reconstructed with a ramp filter at Nyquist cut-off, producing an image resolution of 5.8 mm (full-width half maximum) at the centre of the field of view. A three-dimensional T1-weighted MRI scan for the purpose of co-registration was acquired on 1.0T Philips HPQ MRI scanner [voxel size 1 x 1 x 1.3 mm; 128 contiguous slices; repetition time (TR) 35 ms, echo time (TE) 6 ms, flip angle 35°] on the same day of the PET scan.

Regional binding of $[^{11}\text{C}]$(R)-PK11195 expressed as binding potential (BP), a measure of specific binding of the tracer, was calculated using a basis function implementation of a simplified reference tissue model.[26][27][28] The selection of an anatomically defined reference region may introduce errors since an *a priori* assumption that the chosen reference region is devoid of specific binding has to be made. This is particularly true under conditions where global changes in binding may occur, such as in metabolic encephalopathy. Therefore, cluster analysis [27] [29] was employed as an alternative approach for the extraction of a normal ligand kinetic to serve as the reference input function as previously described.[28] [30] For each patient the appropriate ligand kinetic was selected by Chi-square test (p<0.05) comparison against a population input kinetic previously created from the ligand kinetics of the normal cortex in healthy control patients.[28] [30]

For the calculation of regional mean BP values, the following anatomic volumes of interest (VOIs) were defined on the individuals' volumetric MRI prior to spatial co-registration with the regional $[^{11}\text{C}]$(R)-PK11195 binding potential map [31]: right and left temporal gyrus (superior, inferior and middle), insula, inferior parietal lobule, anterior and posterior cingulate gyrus, dorsolateral prefrontal cortex as well as right and left pallidum, putamen and thalamus. The cerebellum, seen within the restricted field of view (10.65 cm) of the PET camera only to a varying extent, was excluded from formal analysis.

**Statistical analysis**

Student's t-test was used to determine the significance of the differences in regional mean $[^{11}\text{C}]$(R)-PK11195 binding between normal control subjects and patients. Z-values were calculated to test for significant increases in $[^{11}\text{C}]$(R)-PK11195 binding in the brains of individual patients, compared to normal control brain. Since $[^{11}\text{C}]$(R)-PK11195 binding changes are unidirectional (i.e. only increases) a z-value of 1.6 in a one-tailed z-test represents a level of significance of p<0.05.[32] Potential error due to multiple comparisons was examined using the Hochberg correction and p-plot graphical method for the estimation of the number of "true" null hypotheses.[33] Analysis of the set of p values revealed a widespread pattern of statistically significant
changes. The estimated number of 'true' null hypotheses was <3. This being the theoretical default minimum of 'true' null hypotheses, it indicates that correction for multiple comparisons would not be meaningful for this data set.

Results
All patients were clinically normal on examination, but showed slowing of the alpha rhythm in the EEG below the reference range of 8.9 cycles per seconds and/or impaired performance in at least two of the four psychometric tests, compared to the reference range for normal healthy volunteers. None had structural brain abnormalities on T1-weighted MRI, apart from hyperintensity in the basal ganglia in three out of the five patients (patient # 1, 2 and 4), a finding common in chronic liver disease (Figure 1).[34] All patients had cerebral metabolite abnormalities on 1H MRS (increased glutamine/glutamate and reduced choline resonances) and on 31P MRS (reduced phosphomonoester, phosphodiester and β-nucleoside triphosphate resonances), which have previously been described in patients with hepatic encephalopathy. [21,22,23]

With regard to the PET study, group analysis of the patients revealed increases in [11C](R)-PK11195 binding bilaterally in the pallidum (right BP: HE 0.39±0.08; controls 0.27±0.07; p<0.01; left: HE 0.35±0.10; controls 0.25±0.07; p<0.05), in the right putamen (BP: HE 0.31±0.06; controls 0.25±0.04; p<0.05) and the right dorsolateral prefrontal region (BP: HE 0.27±0.08; controls 0.15±0.06; p<0.01) (Figure 2). Individual analysis demonstrated marked heterogeneity in the regional pattern of increased [11C](R)-PK11195 binding (Figures 1 and 3). Three patients (patients 2, 3 and 5) showed a widespread increase in [11C](R)-PK11195 signal, which was particularly pronounced in the pallidum and the frontal cortex (Figure 3). The observation appeared to be unrelated to the aetiology of the disease or other clinical characteristics, apart from the severity of cognitive decline. The three patients (patients 2, 3 and 5) with significantly increased [11C](R)-PK11195 binding were those impaired in the majority of the psychometric tests administered. In contrast, the two patients who were impaired in one test (i.e. NCT B) had either normal regional mean [11C](R)-PK11195 binding in all regions (patient 1) or increased [11C](R)-PK11195 binding only in the anterior cingulate cortex (patient 4) (Figure 3).

Discussion
Increased PK11195 binding has been shown in experimental animal studies [2] and in post-mortem brain samples from patients with HE. Our study is the first attempt to localise and quantify the expression of PK11195 binding sites in vivo in patients with HE.

Regional pattern
Though increases in [11C](R)-PK11195 binding were widespread in some HE patients, group analysis suggested the presence of a regional pattern, whereby the highest level of [11C](R)-PK11195 binding was found in basal ganglia, dorsolateral prefrontal regions and anterior cingulate gyrus. The involvement of the pallidum in liver disease is widely known and T1-weighted MRI signal hyperintensities are well documented and have been ascribed to manganese deposition.[36] Abnormal MRI signal intensities in the pallidum correlate with poor motor performance in tasks involving speed, while cognitive decline is associated with measures of cortical
atrophy.[37] In our study, three out of the five patients showed T1-weighted MRI signal hyperintensities in the pallidum and only one matched with a significant increase in $[^{11}C](R)$-PK11195 binding indicating that increases in PBBS appear to be unrelated to MRI signal alteration.

The regionality detected by $[^{11}C](R)$-PK11195 PET is, instead, in keeping with other neuroimaging observations, such as decreased glucose metabolism and cerebral blood flow in the frontal-limbic-basal ganglia circuits that correlate with the neuropsychologic deficits.[38] [39] It is thus possible that widespread increased $[^{11}C](R)$-PK11195 binding with a superimposed regional pattern involving the basal ganglia and the frontal regions is one pathological correlate of the cognitive impairment found in HE.

Clinical correlations
In our study, neither the duration and the severity of liver disease, expressed as Child-Pugh score, nor the blood ammonia levels correlated with $[^{11}C](R)$-PK11195 binding. However, we found instead that the patients with the highest $[^{11}C](R)$-PK11195 binding were the cognitively most impaired on psychometric testing.

Pathophysiological relevance of increased PK11195 binding
The apparent link between upregulation of $[^{11}C](R)$-PK11195 binding and cognitive decline, the key symptom for minimal HE, supports the hypothesis that PBBS in the brain could indeed participate in the pathogenic mechanism of HE. This further implies that glial cell dysfunction is pivotal in this process, since the binding sites for PK11195 binding are exclusively non-neuronal and, given an intact blood-brain barrier, have been shown to be expressed only by activated or reactive glia (microglia and astrocytes). One theory, the “neurosteroid hypothesis”, suggests that by virtue of the regulatory influence of PBBS on the transport of cholesterol across the mitochondrial membrane,[40] [41] higher expression of PBBS increased the synthesis of neurosteroids, such as pregnenolone. Neurosteroids in turn, are potent positive allosteric modulators of GABA and/or glutamate neurotransmission.[3] Several experimental studies have confirmed that conditions inducing hyperammonaemia result in an increase in PK11195 binding and brain pregnenolone synthesis and are concomitant with the clinical presentation of HE.[42]

Although our study does not resolve the issue of the relative contribution of the different glial subtypes to the increased $[^{11}C](R)$-PK11195 signal in vivo, the binding sites of this ligand have shown to be expressed only by glial cells in a reactive or dysregulated state. There is now a substantial body of evidence that the responses in a wide range of disease conditions of both astrocytes and microglia are accompanied by profound functional changes, such as the expression of cytokines, their respective receptors and other molecules characteristic of an inflammatory tissue response, such as reactive oxygen intermediates. These changes are often referred to as ‘glial inflammation or neuroinflammation’ since they largely occur without any recruitment of blood-borne immune cells seen in classical inflammation.[43] Microglia and the regulatory influence that astrocytes exert over them are increasingly recognised as the cellular link between the peripheral immune system and the local immune system in the brain.[43] Long overlooked, microglial responses to a large variety of pathological stimuli (including subtle toxic ones) are now known to set in early and rapidly.[43] They are regularly found well before overt pathological changes are noted, such as in
cortical spreading depression, a condition that has been referred to as “a pathology without pathology” and even in behavioural changes, such as in repetitive movements, that may be otherwise viewed as physiological.[44] [45]

In conclusion, in the brains of patients with HE that we studied, abnormally high $[^{11}\text{C}] (R)$-PK11195 binding in the frontal lobe was detected in those patients with the poorest performance on psychometric testing. This was independent of underlying aetiology of liver disease and most likely reflects glial activation with concomitant upregulation of PBBS. The observation is in keeping with the hypothesis that PBBS may have a key role in the pathogenesis of hepatic encephalopathy via neurosteroids synthesis. Given the fact that activated, but not resting microglia, (which represent the brain’s intrinsic immune effector cells), are an important source of de novo $(R)$-PK11195 binding, our observation also supports more recent hypotheses that in HE, like in many other primarily neurological conditions, a local inflammatory mechanism within the brain itself may act synergistically with the toxic effects of a noxious agent, which in the case of HE would primarily be ammonia.[14] [15]

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LEGEND

Figure 1

MRI and \([^{11}\text{C}]\)(R)-PK11195 PET images of patients with HE. All images follow the radiological convention: the left side of the image corresponds to subject’s right side. Figure 1 (a and b) transverse and coronal orientation of T1-weighted MRI images (first and third) and co-registered \([^{11}\text{C}]\)(R)-PK11195 images overlaid on MRI (second and fourth) of Patient 1, showing MRI hyperintensity in the basal ganglia and normal \([^{11}\text{C}]\)(R)-PK11195 binding (only constitutive binding in the thalamus). Figure 1 (c and d) show \([^{11}\text{C}]\)(R)-PK11195 PET images of Patient 2, (e and f) Patient 3 and (g and h) Patient 4. Increase in \([^{11}\text{C}]\)(R)-PK11195 binding sites are localized in the frontal lobe, particularly in the anterior cingulate cortex (f-g) and in the white matter along fiber tracts such as in the corpus callosum (c) or following projections connecting the frontal cortex with subcortical structures (e). Figure 1 (h) shows a sagittal view of Patient 4 demonstrating the spatial pattern of increase in \([^{11}\text{C}]\)(R)-PK11195 binding involving the basal ganglia and the frontal lobe (arrows). In Patient 2 (d), MRI hyperintensity in the basal ganglia overlaps with the regions with increase \([^{11}\text{C}]\)(R)-PK11195.

c: corpus callosum; ac: anterior cingulate; wmt white matter tract. The colour scale is calibrated for binding potential values from 0 to 1. White indicates values >1.

Figure 2

Overview of the individual patient’s mean \([^{11}\text{C}]\)(R)-PK11195 binding potential and standard deviation for each region of interest. The aetiology of each patient’s disease is indicated in brackets. The degree of cognitive impairment (cognitive score) is expressed as the number of impaired psychometric tests out of the 4 tested in the battery. Patients 2, 3 and 5 showed a widespread increase in mean \([^{11}\text{C}]\)(R)-PK11195 binding potential (expression of increased PBBS) throughout the cortical and subcortical regions with predominance in frontal areas and basal ganglia. These patients (2, 3 and 5) showed significant cognitive impairment.

Figure 3

Mean \([^{11}\text{C}]\)(R)-PK11195 binding potential values for all volumes of interest drawn for the right (A) and left (B) hemisphere. Mean values for control subjects are expressed as empty histograms with standard deviation bar on the top. Black dots represent individual patient values. *\(P < 0.05\), **\(P < 0.01\).
Fig. 2

Patient 1 (autoimmune) cognitive score: 1/4

Patient 2 (alcohol) cognitive score: 3/4

Patient 3 (hepatitis C) cognitive score: 4/4

Patient 4 (hepatitis C / alcohol) cognitive score: 1/4

Patient 5 (alcohol) cognitive score: 4/4

C = left
□ = right
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